IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Donal O'Shea et al	Confirmation No.: 8300
Application No.: 10/508,754	Group Art Unit: 1626
Filing Date: September 22, 2004	Examiner: Nyeemah Grazier

For: Compounds Useful as Photodynamic Therapeutic Agents

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

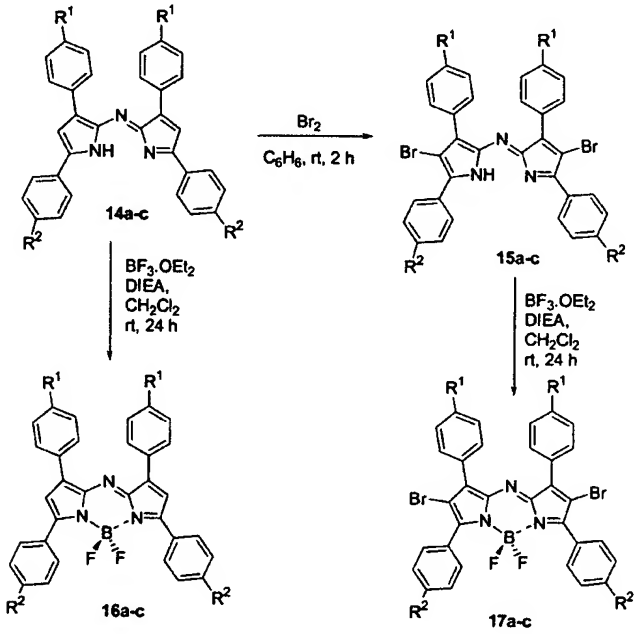
I, Donal O'Shea do hereby declare and say under 37CFR §1.132 the following:

1. I received a B.Sc. and a Ph.D. in Chemistry from University College, Galway, Ireland.
2. I am a Senior Lecturer at University College Dublin, Ireland in the School of Chemistry and & Chemical Biology.
3. I worked in several areas of synthetic organic chemistry from 1990 to the present and direct research on the development of new non-porphyrinic therapeutic window photosensitisers, the BF_2 chelated tetra-aryl-azadipyrromethenes, with applications as Photodynamic Therapeutic Agents for cancerous tumour treatment, as *in vivo* molecular biosensors and in the development of supramolecular photonic therapeutic agents.
4. I am an author on 9 full papers, and 8 scientific meeting presentations, specifically in the area of photodynamic therapeutic agents and BF_2 chelated tetra-aryl-azadipyrromethenes, I am the author of 35 full papers.
5. I am an inventor on the above captioned patent application.
6. I am familiar with the subject matter of this patent application and the recent comments by the examiner regarding this application in the office action dated March 27, 2006.
7. I am familiar with the prior art references cited by the examiner. More specifically, I am familiar with Morgan et al., US 5,446,157; Brinkley, et al., US 5,236,692; WO 93/23492; and Hirosuke, T., JP 11092479;
8. I have conducted, overseen, controlled or reviewed and understand the following studies demonstrating the unexpected and advantageous properties of experimental compositions according to the present invention over the prior art cited by the examiner.

9. SYNTHESIS OF THE COMPOUNDS

(a) Compounds **16a** to **16c** and **17a** to **17c** were prepared, from the respective starting materials **14a** to **14c/15a** to **15c**, by following the methodology taught in Example 1 of the application as filed. Compounds **16a** and **17a** correspond to compound numbers **2a** and **4a** and Compounds **16b** and **17b** correspond to compound numbers **2b** and **4b** described in the application as filed.

Figure 1.



Compound	R^1	R^2
14a	H	H
14b	H	OCH_3
14c	OCH_3	H

10. PHOTOSENSITIZER SPECTROSCOPIC PROPERTIES

(a) The absorption spectra of **16a-c** and **17a-c** in aqueous formulated solution were recorded and show a strong $S_0 \rightarrow S_1$ transition with wavelength of maximum absorbance varying between 651 and 696 nm depending upon substituents (Table 1).

Table 1 Spectroscopic Absorbance Properties of 16a-c and 17a-c[a]

entry	comp.	λ max[b] / nm	λ max[c] / nm	λ max[d] / nm	λ max[e] / nm	fwhm / nm	ε[e] / M^{-1} cm^{-1}
1	**16a**	658	655	647	650	53[b] (49)[e]	79,000
5	**17a**	651	652	645	650	57[b] (47)[e]	79,000
2	**16b**	696	693	686	688	57[b] (55)[e]	85,000
6	**17b**	685	683	675	679	86[b] (57)[e]	75,000
3	**16c**	671	666	660	664	57[b] (57)[e]	78,000
7	**17c**	655	655	646	653	66[b] (57)[e]	80,000

[a] Concentration 5 x 10^{-6} M, rt. [b] H_2O / Cremophor EL(CrEL). [c] Toluene. [d] Ethanol. [e] Chloroform.

(b) Comparisons of **16a-c** with their corresponding di-brominated derivatives **17a-c** show that the introduction of the heavy-atoms gives rise to a moderate hypsochromic shift ranging from 7 to 16 nm. Remarkably, the substitution of bromines onto the pyrrole rings results in only minor changes in the maxima or the shape of the absorption bands. For example, a comparison of **16a** and **17a** in aqueous solution show only a variance of 7 nm in their λ max values (Table 1, entries 1, 5). This demonstrates that the heavy-atom can be introduced without diminishing the advantageous absorption characteristics of the photosensitizers of the present invention.

(c) The fluorescence properties of the sensitizers were examined in aqueous formulated solutions, toluene, ethanol and chloroform. Excitation of the compounds **16a-c** and **17a-c** in aqueous solutions at 630 nm all gave fluorescence bands which were mirror images of the absorbance spectra with Stoke shifts in the range of 22 to 38 nm (Table 2). The compounds **16a-c** showed a range of high fluorescence quantum yields (Φ_f) measured in chloroform from 0.23-0.36 (Table 2). In comparison, the introduction of bromine directly into the core of the photosensitizer gave rise in each case to substantial reduction in fluorescence

quantum yields for **17a-c** (Table 2, entries 5, 6, 7) indicating that, when a heavy atom such as bromine is directly substituted onto the central core of the photosensitizer, a heavy-atom effect can be induced which, depending upon other possible competing photophysical pathways, may translate into increased singlet oxygen production.

Table 2. Spectroscopic Fluorescence Properties of 16a-c and 17a-c[a]

entry	comp.	λ max[b] / nm	Stoke shift[b] / nm	λ max[c] / nm	λ max[d] / nm	λ max[e] / nm	Φ_f[e]
1	**16a**	683	25	676	669	672	0.34
5	**17a**	679	28	672	666	673	0.012
2	**16b**	727	31	717	715	715	0.36
6	**17b**	719	36	714	712	714	0.10
3	**16c**	701	30	693	697	695	0.23
7	**17c**	693	38	683	680	679	<0.01

[a] Concentration 2 x 10^{-7} M, rt. [b] H_2O / CrEL. [c] Toluene. [d] Ethanol. [e] Chloroform.

11. X-RAY STRUCTURE OF 17a

The X-ray structure of **17a** provides solid state evidence for a brominated derivative, the patent application already providing a comparative crystal structure for a non-brominated example.

(a) The introduction of heavy-atoms with large atomic radii into a photosensitizer can result in structural deformation to the planarity of the molecule. In order to assess whether structural deformation had occurred, the sensitizer structure imparted by the bromine atoms in **17a-c**. **17a** was crystallized. This was achieved by the slow room temperature evaporation of a toluene solution, in the monoclinic space group Cc (#9) with four molecules in the unit cell. A thermal ellipsoid drawing of **17a** is shown below:

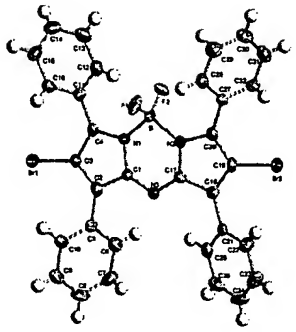


Figure 2. Perspective drawing of 17a. Thermal ellipsoids drawn at 80% probability level.

(b) In spite of the introduction of two bromine atoms onto the sensitizer **17a**, the planarity of the central 12-atom core of the molecule was preserved. The bromine bond angles from pyrrole ring 1 (N1/C1/C2/C3/C4) are -0.126(1) Å for Br(1) and 0.109(1) Å for Br(2) from planarity and for pyrrole ring 2 (N2/C17/C18/C19/C20) Br(1) is –0.415(1) Å and Br(2) is 0.004 Å. The angle of intersection of pyrrole ring 1 and pyrrole ring 2 is small at 4.2(2) degrees. The 12-atom plane of the central tricyclic structure shows the greatest deviation from this plane of 0.100(2) Å for the atom N(1). Unexpectedly, this structure compares favorably with the structure of non-brominated **16b** reported in Figure 3 of the application as filed, which has a comparable intersect angle of pyrrole ring 1 and pyrrole ring 2 of 4.1(3) degrees. Additionally, the central tricyclic 12-atom plane of **16b** shows the greatest deviation from this plane of 0.087(3) Å for the atom N(2). These results support the supposition that introduction of heavy atoms such as bromines at β-pyrrole position of **17a-c** could give rise to a more efficient population of the triplet state without causing an increase in non-radiative decay to the ground state.

12. COMPARATIVE STUDY OF SINGLET OXYGEN GENERATION IN SOLUTION WITH LIGHT > 600 NM

(a) The ability to gain control over singlet oxygen production by exploiting the heavy-atom effect, was tested by carrying out a comparative singlet oxygen generation analysis. The study was undertaken by monitoring the reaction of the singlet oxygen acceptor 1,3-diphenylisobenzofuran (DPBF) with photosensitizer generated singlet oxygen by following the disappearance of the 410 nm absorbance band of DPBF at initial concentration of 5 x 10^{-5} M over a time period of 1 h. Each of the sensitizers **16a-c** were examined at a

concentration of 5 x 10^{-6} M and compared to hematoporphyrin as a reference sensitizer. The pyrrole brominated derivatives **17a-c** were examined at a lower concentration of 5 x 10^{-8} M and compared to the reference sensitizer methylene blue. Relative rates of oxygenation of DPBF by **16a-c** and **17a-c** versus hematoporphyrin and methylene blue were estimated by comparison of the rates of consumption of DPBF at the initial stages of each experiment. The standard sensitizers (hematoporphyrin and methylene blue) have singlet oxygen quantum yields of 0.65 and 0.50, respectively, in methanol.

Table 3. Comparative singlet oxygen generation of **16a, 16b, 16c**, hematoporphyrin at 5 x 10^{-6} M concentration.

Compound	**1***	**16a**	**16b**	**16c**
rel. rate	1	0.46	0.4	1.2

*hematoporphyrin

Table 4. Comparative singlet oxygen generation of 17a, 17b, 17c, methylene blue at 5 x 10^{-8} M concentration.

Compound	**9***	**17a**	**17b**	**17c**
rel. rate	1	2.9	7.7	4.2

*methylene blue

(b) A heavy-atom effect can be observed when R^2 and R^5 is an alkyl, cyclic or heterocyclic moieties with heavy atom substitutions, so that the heavy atom (such as bromine) is not incorporated directly on the central core of the photosensitizer. All of **17a-c** showed an increased efficiency of singlet oxygen generation in comparison to **16a-c**, even at a one-hundred fold lower concentration of 5 x 10^{-8} M. The dramatically enhanced singlet oxygen production levels of **17a-c**, when contrasted with **16a-c**, show that the inclusion of the heavy-atom as a substituent directly onto the central core of the photosensitizer results in singlet oxygen production without given rise to loss of excited state energy by internal radiationless transitions. No significant photobleaching of the sensitizers was observed during these experiments.

13. LIGHT INDUCED CYTOTOXICITY ASSAY

(a) Two different cell types were examined in the assay, MRC5-SV40 transformed fibroblast cells and HeLa cells. Varying concentrations of Cremophor EL formulated aqueous solutions of the photosensitizers were incubated with the cells in the dark for 3 h. Subsequently, the culture medium was removed and fresh culture medium added to each well. The plates were irradiated using a light source of wavelength 600-750 nm delivering a light dose of either 8 or 16 J cm^{-2}. Following irradiation, the cells were incubated for a further 48 h at 37 °C, after which time, percentage cell viability was determined using a tetrazolium chlorimetric reduction assay. Dark toxicity of photosensitizers was determined by carrying out an identical experiment as described above except that the light irradiation step was omitted (0 J cm^{-2}). All assay experiments were carried out in triplicate and an average of the three individual runs are presented. Hematoporphyrin was used as a comparative standard control and was assayed according to previously documented procedures.

(b) MRC5-SV40 cells displayed no determinable dark toxicity with **16b, 17b** or hematoporphyrin up to a concentration of 10^{-4} M, Table 5. In contrast, irradiation with 8 J cm^{-2} light dose showed a significant light induced toxicity with EC-50 values determined for **16b** and **17b** as 1.1×10^{-4} and 3.7×10^{-8} M, respectively (Table 5). The exceptional light induced toxicity of **17b** was very encouraging as this molecule contained the two bromine heavy atoms directly substituted onto the core of the photosensitizer.

(c) As phototoxicity should be dependent upon light dose as well as photosensitizer concentration, the assay was repeated with a higher light irradiation of 16 J cm^{-2}. The higher light dose resulted in an improved EC-50 value for each of our studied photosensitizers with values obtained for **16b** at 1.7×10^{-5} M, and **17b** at 1.4×10^{-8} M (Table 5). Each of the tested photosensitizers, performed better at this light dose than the standard control hematoporphyrin (Table 5).

Table 5. In vitro EC-50 Assay Data for MRC5-SV40 Cells[a]

entry	comp.	EC-50 (M) / 8 J cm^{-2}	EC-50 (M) / 16 J cm^{-2}
1	**1***	6.3 (±3) x 10^{-5}	3.7 (±1) x 10^{-5}
3	**16b**	**1.1 (±1) x 10^{-4}**	**1.7 (±1) x 10^{-5}**
5	**17b**	**3.7 (±0.3) x10^{-8}**	**1.4 (±0.1) x10^{-8}**

[a] Standard deviation in brackets *hematoporphyrin

(d) The data portray how the compounds covered by the pending claims unexpectedly exhibit a remarkable spectrum of activity (from the micro- to nano-molar range) across these structurally related sensitizers. In the case of **17b**, exploitation of the heavy-atom effect in vitro is seen to be a viable method to control the excited triplet state population and singlet oxygen quantum yields, as well as to translate that control into significantly greater in vitro efficacy. **While in structural terms 16b and 17b only differ by the two bromine substituents, this gives rise to a divergence in efficacy by over a 1000 fold.**

(e) A second study was carried out using the HeLa cell line. Photosensitizer dark toxicity was only observed at high concentrations for **16b**, with the most active compound **17b**, showing no observable dark toxicity in the tested concentration range (Table 6). A broad range of light induced cytotoxicity was also observed for the photosensitizer series in HeLa cells which was comparable to that observed in the MRC5-SV40 cell line. Determined EC-50 values for the series with a light dose of 16 J cm^{-2} showed that **16b** (3.1 x 10^{-5} M) was the least active in the assay, the best being **17b** (4.1 x 10^{-8} M) (Table 7). An improvement in efficacy for each photosensitizer was observed on increasing the light dose from 8 to 16 J cm^{-2}, again showing the expected light-dose response behavior. Again, the in vitro heavy-atom effect was clearly observed, when comparing EC-50 data for **16b** and **17b** at a light dose of 8 J cm^{-2}, there was over a 1000-fold efficacy increase while, at 16 J cm^{-2}, there was greater than **a 750 fold increase** (Table 6).

Table 6. In vitro EC-50 Assay Data for HeLa Cells[a]

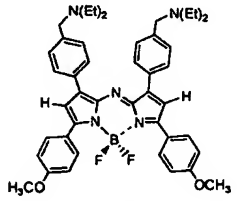
entry	compound	EC-50 (M) / 0 J cm^{-2}	EC-50 (M) / 8 J cm^{-2}	EC-50 (M) / 16 J cm^{-2}
1	**1**	none	3.3 (±2) x 10^{-5}	1.9 (±0.4) x 10^{-5}
2	**16b**	1.1 (±1) x 10^{-4}	**7.2 (±5) x 10^{-5}**	**3.1 (±1) x10^{-5}**
3	**17b**	none	**6.3 (±2) x 10^{-8}**	**4.1 (±3) x 10^{-8}**

[a] Standard deviation in brackets

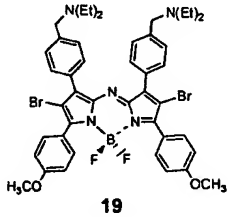
14. Further Examples of the Effect of Heavy Atom Substitution

The compounds in Figure 3 were synthesized and tested.

Figure 3. Further Heavy-Atom Efficacy Comparison.



18



19

(a) The compounds including a heavy atom unexpectedly result in a very large differential in efficacy between the heavy-atom functionalized example (19), when compared to the non-heavy atom substituted derivative (18), with a **480 fold efficacy difference** recorded (Table 7).

Table 7. pKa in H_2O/CrEL,[a] Acid Enhanced Singlet Oxygen Generation[b] and in vitro MRC5 cell line EC_{50} assay data[c] for 18 and 19.

Entry		pKa	$^1O_2$ acidic rate increase	EC_{50} x 10^{-6} (M) MRC5 cell 0 J cm^{-2}	16 J cm^{-2}
1	**18**	6.6	9.2	> 100	2.8
2	**19**	6.6	10.6	4.5 (± 0.7)	0.0058 (±0.003)

[a] pKa measured by fluorescence titration in H_2O/CrEL solutions. [b] rate of singlet oxygen generation in acidic DMF / rate in DMF. [c] light dose of either 0 or 16 J cm^{-2} with standard deviation in brackets.

15. Thus, compounds covered by the claims 27 and 37-58, as set forth in our amendment dated July 27, 2006, in which R^2 and R^5 are a heavy atom or an alkyl, cyclic, or heterocyclic moiety each substituted with at least one heavy atom, unexpectedly result in more efficacious PDT relative to the compounds of the prior art, in which R^2 and R^5 are hydrogen, by increasing the population of T1 and increasing the generation of ground state oxygen while limiting the non-radiative internal back-conversion from T1 to the ground state S0 and the inhibition of the photosensitizer triplet to ground state oxygen energy transfer.

I, Donal O'Shea further declare that all statements made herein are true to the best of my knowledge, or if made upon information and belief, are believed to be true. This Declaration is made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 US §1001, and may jeopardize the validity of the subject patent application or any patent issuing thereon.

Respectfully submitted,

15-Oct-2006
Date

Donal O'Shea
Donal O'Shea